DOCKET NO. JJM-399

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Michael W. Grady et al.		
Serial No.:	09/254,407	Art Unit: 1623	
Filed :	February 7, 2000	Examiner: E. White	
For :	SULFATED POLYSACCHARIDES AND USES THEREOF IN MEDICAL TREATMENT		

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March 16, 2004
(Date)

Theodore J. Shatynski
Name of applicant, assignee, or Registered Representative

Theodore J. Shatynski
(Signature)

March 16, 2004
(Date of Signature)

AUTHORIZATION TO CHARGE DEPOSIT ACCOUNT

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Dear Sir:

Attached is an Appeal Brief for the above-captioned patent application.

Please charge Deposit Account No. 10-0750/JJM-399/TJS in the name of Johnson & Johnson in the amount of $320.00, representing the cost of filing a Brief on Appeal in the above-captioned matter.

The Commissioner is hereby authorized to charge any additional fees which may be required to Account No. 10-0750/JJM-399/TJS. This Authorization is being submitted in triplicate.

Respectfully submitted,

Theodore J. Shatynski

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DATED: March 16, 2004

425

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ATTENTION: BOARD OF PATENT APPEALS AND INTERFERENCES

APPELLANTS' BRIEF (37 C.F.R. 1.192)

This is an appeal from the final rejection mailed February 14, 2003, a Notice of Appeal having been received by the USPTO August 13, 2003. Appellants' Brief is being submitted on March 12, 2004, concurrently with a Petition for Revival for Unintentional Abandonment uder 37 CFR 1.137(b).

The fees required under Section 1.17(f), and any required petition for extension of time for filing this brief and fees therefor, are addressed with in the accompanying TRANSMITTAL OF APPEAL BRIEF.

This brief is transmitted in triplicate. (37 CFR 1.192(a))

This brief contains these items under the following headings, and in the order set forth below (37 CFR 1.192(c)):

TABLE OF CONTENTS

1. REAL PARTY INTEREST

The real party in interest of the subject patent application is Ethicon, Inc. having a principal place of business at U.S. Route #22, Somerville, New Jersey 08876.

2. RELATED APPEALS AND INTERFERENCES

There are no related appeals or interferences pending.

3. STATUS OF CLAIMS

3.1 Claims 2-10 stand rejected under 35 U.S.C. §112, second paragraph.

3.2 Claims 1-10 and 13 stand rejected under 35 U.S.C. §103(a) as unpatentable over EP 140,596 (Easton) in view of U.S. 3,939,836 (Tunc).

3.3. Claims 1-10 and 13-15 stand rejected under 35 U.S.C. §103(a) as being unpatentable over U.S. 5,679,375 (Spilburg).

3.4 Claims 11 and 12 are objected to under 37 CFR 1.75(c) as being improper form because a multiple dependent claim cannot depend from any other multiple dependent claim.

4. STATUS OF AMENDMENTS

An amendment after Final Rejection has been filed and indicated as entered by the Examiner in the Advisory Action mailed July 3, 2003.

5. SUMMARY OF INVENTION

The present invention is directed toward a wound dressing or ointment comprising a synthetic sulfated polysaccharide selected from the group consisting of sulfated cellulose

derivatives and sulfated polyanionic polysaccharides, said synthetic sulfated polysaccharide being present in an amount sufficient to bind matrix metalloproteinases. (Claim 1)

It has been found surprisingly, that the sulfated polysaccharides according to the present invention have an exceptional ability to bind to matrix metallproteinases (MMP's) (Spec., page 5, lines 15-18). Such matrix metalloproteinases are implicated in a number of medical conditions, including chronic wounds such as decubitis ulcers (Spec., page 5, lines 18-20). This is because the balance between matrix deposition and tissue turnover, which in turn may depend on the balance between proteolytic enzymes and their inhibitors, is fundamental to wound healing and other medical conditions (Spec., page 5, lines 20-24). Chronic wound fluids have been shown to contain elevated levels of MMP2 (Gelatinase A) and MMP9 (Gelatinase B). (Spec., page 5, lines 24-26).

6. STATEMENT OF ISSUES

6.1 Whether claims 2-10 are unpatentable under 35 U.S.C. §112, second paragraph.

6.2 Whether claims 1-10 and 13 are unpatenable under 35 U.S.C. §103(a) as unpatentable over EP 140,596 (Easton) in view of U.S. 3,939,836 (Tunc).

6.3 Whether claims 1-10 and 13-15 are unpatentable under 35 U.S.C. 103(a) over U.S. 5,679,375 (Spilburg).

6.4 Whether claim 11 and 12 are in improper multiple dependent form under 37 CFR 1.75(c).

7. GROUPING OF CLAIMS

For the purpose of the appeal, the following groups of claims do not stand or fall together.

7.1 Group I, includes claims 1-10 directed toward a wound dressing or ointment comprising a synthetic sulfated polysaccharide selected from the group consisting of sulfated cellulose derivatives and sulfated polyanionic polysaccharides, said synthetic sulfated polysaccharide being present in an amount sufficient to bind matrix metalloproteinases.

7.2 Group II, includes claims 12 and 13 directed toward a pharmaceutical composition comprising a sulfated polysaccharide as in any of the claims 1 to 7.

7.3 Group III, includes claims 14 and 15 directed toward treatment of medical conditions rediated by a matrix metalloproteinase wherein the treatment comprises applying to a wound the composition of claim 1.

7.4 Group IV, includes claim 11 relting to a wound dressing or ointment wherein the sulfated polysaccharide is soluble in water to an extent of at least 10 g/l at 25°C.

Groups I, II, III and IV are separately patentable. Group III is separately patentable from Groups I, II, and IV as Group III is directed to a method of treatment of medical conditions mediated by matrix metalloproteinase. The articles claimed in Groups I, II and IV may be used in other methods than those claimed in Group IV. Group II is separately patentable over Groups II, III and IV, as Group II excludes compositions that are not pharmaceuticals as claimed by

Group II. Group IV is separately patentable over the articles claimed in Groups I and II and the method of Group II as Group IV excludes other articles and methods not having solubility in water to an extent of at least 10 g/l at 25°C.

Therefore, the claims of Groups I, II, III, and IV are separately patentable from among themselves.

8. ARGUMENTS

8.1 Claims 2-10 are definite under 35 U.S.C. §112, second paragraph.

Appellants believe their amendments under 37 CFR 1. 116 have addressed the Examiner's concerns presented at Final Rejection. Therefore, this §112 rejection is respectfully requested to be reversed.

8.2 Claims 1-10 and 13 are not obvious over EP 140,596 (Easton) in view of U.S. 3,939,836 (Tunc).

Easton is directed to complexes of polyanionic plant polysaccharides with biodegradable proteins or proteolytic degradation products thereof and are useful in the formation of wound dressings and surgical implants, such as sutures, blood vessel grafts and artificial organs. The biodegradable protein part of the complex is preferably collagen and the preferred polysaccharide is alginate. Sulphate dextrans appear to be the only example of a polyanionic plant polysaccharide provided among those of carrageenans, celluloses such as carboxymethyl cellulose, xanthan gum and sulphate dextrans. Nothing is disclosed about any special properties of sulphate dextrans among the other examples of disclosed polysaccharides.

Tunc discloses sulfated cellulose esters used as binders in nonwoven fabrics.

Easton, while disclosing various forms of polysaccharides, contains no indication that the sulfated polysaccharides of the present invention are superior to other non-sulfated polysaccharides in binding MMP's as demonstrated by Applicant's comparative data (Please note Fig. 1 and Fig. 2 of Appellant's specifications (attached as Appendix B) and the discussion in the specification regarding the superiority of sulfated polysaccharides in binding MMP's, e.g., Procedures 1 and 2 of Example 5).

From Tunc it is not apparent why the claimed wound dressings or ointments which bind MMP's would be obvious. More specifically Tunc is silent with regard to the claimed wound dressing or ointment, but more importantly Appellants have demonstrated that the sulfated polysaccharides of the present invention are superior in binding MMP which have been implicated in preventing the healing of chronic wounds (See Appendix C article "Wound Fluid from Chronic Leg Ulcers Contains Elevated Levels of Metalloproteinases MMP-2 and MMP-9" by Wysocki et al; please note distinction between "chronic" wounds and "acute" wounds.). Since Tunc is not concerned with treatment of chronic wounds and binding of MMP's.

Based on the foregoing, the cited art does not teach or suggest the surprising ability of sulphated polysaccharides in binding MMP's as compared to other polysaccharides. Therefore the Examiner's rejection is respectfully requested to be reversed.

8.3 Claims 1-10 and 13-15 are not obvious over U.S. 5,679,375 (Spilburg)

Spilburg is concerned with a composition for treating gastric or duodenal ulcers using sulfated polysaccharides which are non-absorbable in the gastro-intestinal tract. (claim 1 and column 2, lines 45-54.)

Appellants submit that it is not obvious to use something for internal bodily treatment of gastric ulcers for topical wound dressing or ointment applications. Please note that internal gastric ulcers are quite different from topical chronic ulcers that wuld be treated by a wound dressing or ointment; i.e. proteases found in the chronic ulcer environment compared to proteases found in the gastric environment are quite different. For example, proteases found in gastric ulcers are acidic (pH $\simeq$2) and therefore are only active at low pH. However, in the case of chronic ulcers, the pH of the ulcer is approximately 7, and the MMP's found in the chronic wounds are only active in the pH range of about 6-8. Thus, due to the widely different applications and environments between those disclosed in Spilburg and those of Appellant's invention, it is respectfully submitted that this rejection is improper and Appellants, therefore, respectfully request its reversal.

8.4 Claims 11 and 12 are in proper multiple dependent form

MPEP § 608.01(n) provides examples of acceptable multiple dependent claim wording. Under subheading A, the following acceptable example are provided:

> "Claim 5. A gadget as in any one of the preceeding claims, in which ..."
>
> "Claim 5. A gadget as in any one of claims 1, 2, and 3, in which ..."

These acceptable formats are analogous to the multiple dependent format of claims 11 and 12. Specifically, both claims 11 and 12 contain analogous claim language of ..." as in any one of claim 1 to 7 wherein ...." It is submitted that the analogous language of claims

11 and 12 are not of improper multiple dependent format and the Examiner's objection is respectfully requested to be reversed.

8.6 CONCLUSION

For the foregoing reasons, the reversal of the rejections and objections relating to claims 1-15 are respectfully requested.

9. APPENDIX OF CLAIMS INVOLVED IN THE APPEAL

(See attached)

Respectfully submitted,

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Dated: March 16, 2004

JJM-399

APPENDIX OF CLAIMS INVOLVED IN THE APPEAL

1. (Previously Amended) A wound dressing or ointment comprising a synthetic sulfated polysaccharide selected from the group consisting of sulfated cellulose derivatives and sulfated polyanioninc polysaccharides, said synthetic sulfated polysaccharide being present in an amount sufficient to bind matrix metalloproteinases.

2. (Previously Amended) A wound dressing or ointment comprising the sulfated polysaccharide according to claim 1, wherein the sulfated polysaccharide is selected from the group consisting of sulfated hydroxyethyl cellulose, sulfated carboxymethyl cellulose and sulfated oxidized regenerated cellulose.

3. (Previously Amended) A wound dressing or ointment comprising the sulfated polysaccharide according to claim 1 wherein the sulfated polysaccharide is sulfated oxidized regenerated cellulose.

4. (Previously Amended) A wound dressing or ointment comprising the sulfated polysaccharide according to claim 1 wherein the sulfated polysaccharide is, selected from the group consisting of sulfated alginates, sulfated pectins and sulfated hyaluronic acid.

5. (Previously Amended) A wound dressing or ointment comprising the sulfated polysaccharide according to claim 1 wherein the sulfated polysaccharide is a sulfated alginate.

6. (Previously Amended) A wound dressing or ointment comprising the sulfated polysaccharide according to any preceding claim, wherein the sulfated polysaccharide comprises an average of at least 0.1 sulfate groups for each saccharine residue of the polysaccharide.

7. (Previously Amended) A wound dressing or ointment comprising the sulfated polysaccharide according to claim 6, wherein the sulfated polysaccharide comprises an average of at least 1 sulfate group for each saccharine residue of the polysaccharide.

8. (Previously Amended) A wound dressing or ointment comprising the sulfated polysaccharide according to claim 1, wherein the sulfated polysaccharide has an average molecular weight in the range 25,000 - 250,000.

9. (Previously Amended) A wound dressing comprising the sulfated polysaccharide according to claim 1, wherein the wound dressing is in the form of a woven, non-woven, sponge or knitted fabric.

10. (Previously Amended) A wound dressing comprising the sulfated polysaccharide according to claim 1, wherein the wound dressing is in the form of a solid complex with collagen.

11. (Previously Amended) A wound dressing or ointment comprising the sulfated polysaccharide as in any one of claims 1 to 7 wherein the sulfated polysaccharide is soluble in water to an extent of at least 10g/l at 25°C.

12. (Previously Amended) A pharmaceutical composition comprising a sulfated polysaccharide as in any one of claims 1 to 7.

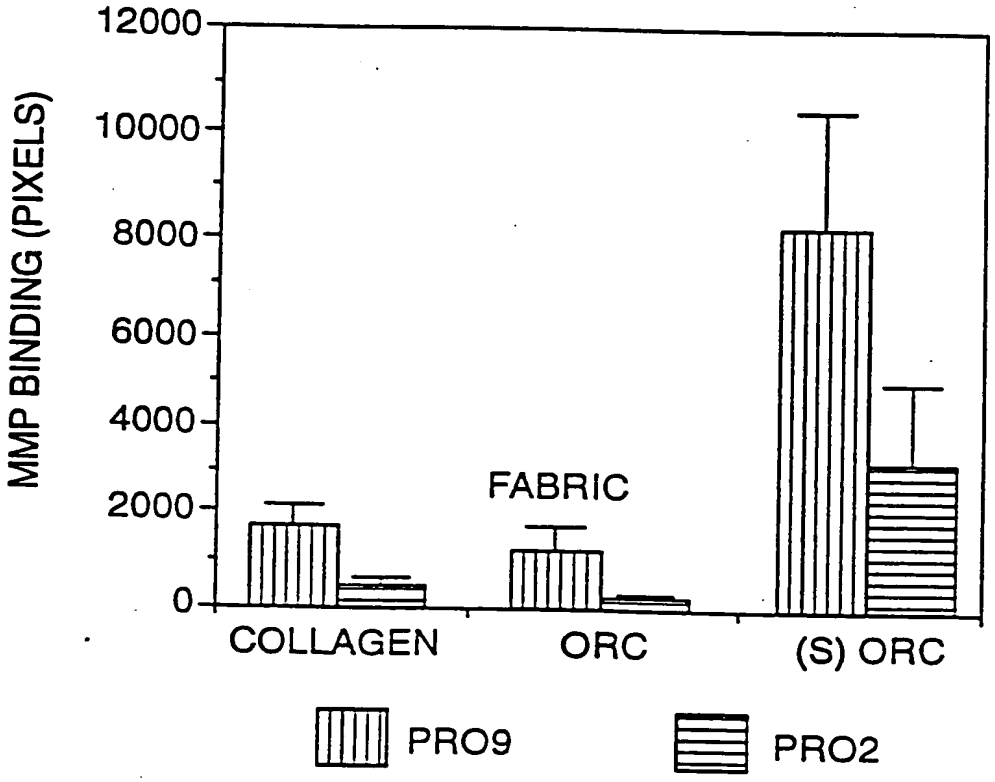
13. (Original) A pharmaceutical composition according to claim 12 in the form of a wound dressing material or a soft tissue implant.

14. (Previously Amended) A method of treatment of medical conditions mediated by a matrix metalloproteinase wherein the treatment comprises applying to a wound the composition of claim 1.

15. (Previously Amended) The method according to claim 14, wherein the medical condition is a chronic wound.

16. (Canceled)

FIG. 1


MMP BINDING (PIXELS)
12000
10000
8000
6000
4000
2000
0
COLLAGEN
FABRIC
ORC
(S) ORC
PRO9
PRO2

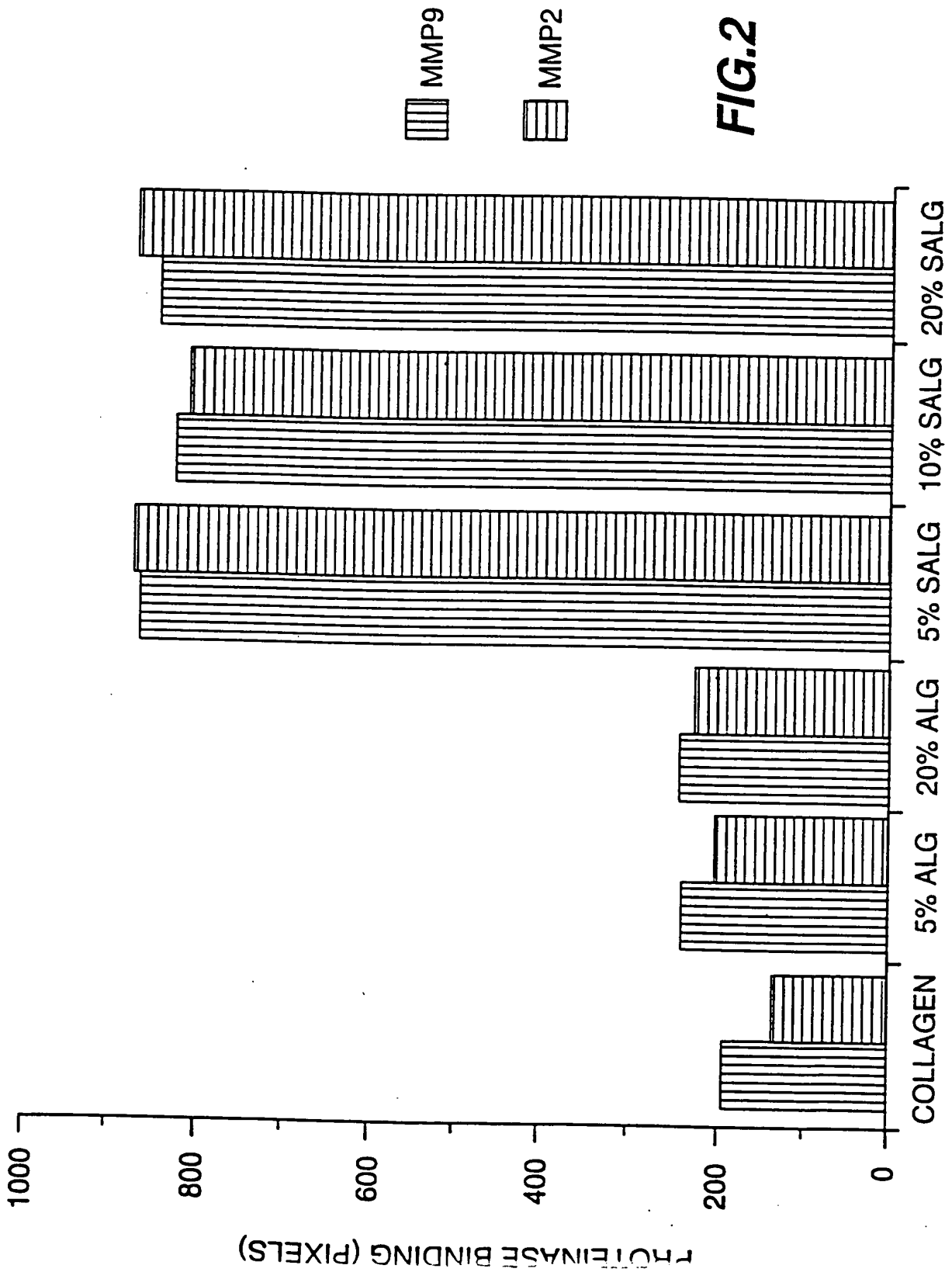

MMP9
MMP2
1000
800
600
400
200
0
PROTEINASE BINDING (PIXELS)
COLLAGEN
5% ALG
20% ALG
5% SALG
10% SALG
20% SALG
DRESSING

FIG.2

Wound Fluid from Chronic Leg Ulcers Contains Elevated Levels of Metalloproteinases MMP-2 and MMP-9

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The purpose of the present research was to determine if metalloproteinase levels were elevated in human chronic wound fluid. Samples of blood and wound fluid from acute (mastectomy) and chronic (leg ulcer) wounds were collected, and metalloproteinase profiles of the samples were determined by gelatin zymography. Compared to serum, acute wound fluid (mastectomy fluid) contained markedly increased levels (five- to tenfold) of several metalloproteinases including 72-kDa and 94-kDa gelatinases (MMP-2 and MMP-9). In chronic wound fluid, not only were these enzyme levels increased another five- to tenfold over mastectomy fluid, but also both activated enzyme and proenzyme species appeared to be present. Our results suggest that non-healing ulcers develop an environment containing high levels of activated metalloproteinases, which may result in chronic tissue turnover and failed wound closure. *J Invest Dermatol 101:64–68, 1993*

Chronic skin ulcers are wounds that fail to heal [1,2]. To learn more about the defect(s) in these wounds, we began analyzing would fluid collected from beneath occlusive dressings as an indicator of the wound environment [3,4]. Our studies showed that degradation of the adhesion proteins fibronectin and vitronectin occurred in chronic ulcers. Degradation was limited to the wound site since there was no evidence of adhesion protein degradation in blood samples obtained from the patients. Moreover, adhesion proteins were intact in fluid obtained from acute wounds (suction blister or mastectomy). Because fibronectin and other adhesion proteins are important for normal cutaneous repair [5], degradation of the adhesion proteins in chronic ulcers could contribute to poor healing of these wounds.

Degradation of adhesion proteins in chronic skin ulcers might occur as a result of uncontrolled proteinase activity in the wound bed. Little is known, however, about proteinase activity in human epidermal wounds. Elevated proteinase levels have been detected in blister fluid from bullous skin diseases [6,7] and corneal ulcers [8–10]. Also, immunostaining of tissue sections with antibodies against interstitial collagenase showed elevated enzyme levels in active scar tissue [11].

Interstitial collagenase is part of a group of enzymes known collectively as the matrix metalloproteinases. These zinc-dependent enzymes, including collagenases, gelatinases, and stromolysins, have homologous structures, are secreted in the form of latent proenzymes, and play important roles in various aspects of normal tissue repair and remodeling [12–15]. Several of the matrix metalloproteinases, including gelatinase (type IV collagenase), have been shown to degrade fibronectin and other adhesion proteins *in vitro* [14,15]. To learn more about the possible role of these proteinases in non-healing ulcers, we collected would fluid samples from acute and chronic wounds, and analyzed proteinase profiles by gelatin zymography. Our results show that metalloproteinase levels were markedly increased in chronic wound fluid compared to acute wound fluid and that chronic wounds contain both proenzyme and activated enzyme species.

EXPERIMENTAL DESIGN

Collection and Preparation of Wound Fluids Informed consent was obtained from individual subjects for all procedures. Subjects with chronic leg ulcers or who had undergone surgical mastectomy operations were recruited from in-patient and out-patient populations of large metropolitan hospitals in Dallas and New York City. Detailed descriptions of the procedures for wound fluid collection and storage and methods for preparation of serum from plasma or blood have been reported previously [3,4]. The subject population used for these studies included six leg ulcer patients, ten mastectomy patients, and four normal volunteers. Leg ulcer patients, two women and four men, ranged in age from 37 to 79; mastectomy patients ranged in age from 39 to 71; and normal volunteers, two women and two men, ranged in age from 32 to 35.

Gelatin-Zymography Metalloproteinase profiles were determined by zymography using gelatin-containing acrylamide gels (8% acrylamide and 4.75 mg/ml gelatin) [16–18]. Samples of chronic wound fluid, mastectomy wound fluid, blood-derived serum (BDS), and plasma-derived serum (PDS) were subjected to sodium dodecylsulfate–polyacrylamide gel electrophoresis (SDS-PAGE) [19] under non-reducing conditions. Each lane was loaded with ~75 μg of protein [20] unless indicated otherwise. After electrophoresis, gels were washed twice with 2.5% Triton-X-100 for

Manuscript received November 19, 1992; accepted for publication February 22, 1993.

Reprint requests to: A. B. Wysocki, Department of Nursing, NYU Medical Center, New York, NY 10016 (correspondence to: F. Grinnell, Department of Cell Biology and Neuroscience, UT Southwestern Med Ctr, Dallas, TX 75235).

Abbreviations: AEBSF, aminoethylbenzenesulfonylfluoride; BDS, blood-derived serum; MMP, matrix metalloproteinase; PDS, plasma-derived serum.

0022-202X/93/$06.00

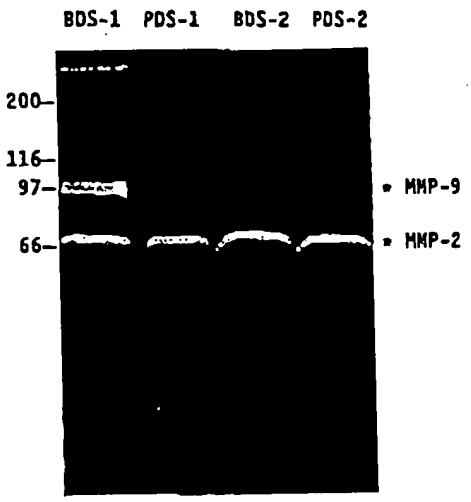

Figure 1. Gelatinase activity in serum derived from blood and plasma. Samples (75 μg) of BDS and PDS were subjected to gelatin zymography. Results shown from two different subjects are representative of the seven subjects studied. A 72-kDa gelatinase (MMP-2 proenzyme) was the predominant gelatinase in PDS, whereas BDS contained significant amounts of a 94-kDa gelatinase (MMP-9 proenzyme) as well as MMP-2.

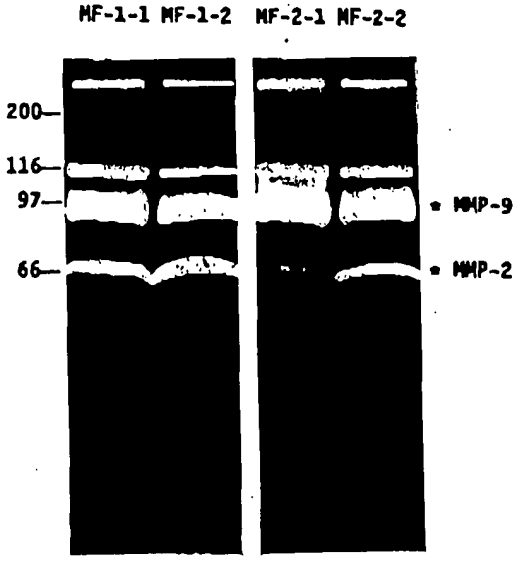

Figure 2. Gelatinase activity in mastectomy wound fluid. Samples (75 μg) of mastectomy wound fluid collected from patients 1 and 2 d after surgery were subjected to gelatin zymography. Results shown from two different subjects are representative of the 10 subjects studied. Compared to serum, mastectomy fluid contained elevated levels of MMP-2 and MMP-9 as well as putative 130-kDa and 225-kDa MMP-9 complexes.

30 min to remove SDS. They were then rinsed briefly with H_2O followed by incubation overnight at 37°C in reaction buffer containing 50 mM Tris-HCl, 150 mM NaCl, and 5 mM $CaCl_2$ (pH 7.4). In studies to determine enzyme specificity, proteinase inhibitors were added to wash or reaction buffer solutions as indicated. At the end of the incubations, the gels were stained with 0.1% Coomassie brilliant blue and destained. Areas of proteinase activity appeared as clear zones against a dark blue background. Bio-Rad high molecular weight standards were used for molecular mass markers.

RESULTS

Gelatinase Activity in Serum Derived from Blood and Plasma Because the overall protein profile of wound fluid was found to be similar to serum [3,4], we examined the gelatinase profile of serum to assess baseline levels of proteinases that might occur in wound fluid. Serum was prepared both from plasma (PDS) and from blood (BDS), making it possible to distinguish between circulating gelatinases and gelatinases released from blood cells during clot formation.

Figure 1 shows representative zymograms for two of four subjects. As indicated in *Materials and Methods*, zymograms utilize gelatin co-polymerized with acrylamide. After removal of SDS from the gels, the gelatin can be degraded by gelatinases that are present in the samples. When the gels are subsequently stained for protein, clear areas in the otherwise dark background identify the location of enzymatic activity. Because the active site of latent metalloproteinases becomes available after SDS treatment, this method detects gelatinases even if they are still in the proenzyme form [17].

PDS contained primarily a 72-kDa gelatinase, a finding consistent with previous studies [21]. Blood-derived serum contained 72-kDa gelatinase and also a 94-kDa gelatinase, indicating that the latter was released from blood cells during clotting. Based on their molecular masses, the 72-kDa and 94-kDa gelatinases correspond to the proenzyme forms of the enzymes designated MMP-2 and MMP-9 [22]. Also, less prominent 130- and 225-kDa gelatinases were detected. These bands probably represented complexes between the 94-kDa gelatinase and other molecules (e.g., metalloproteinase inhibitors). Such complexes have been shown previously to be released by neutrophils [23] and monocytes [24].

Gelatinase Activity in Mastectomy Wound Fluid The above results established baseline gelatinase profiles in serum. We also studied gelatinase profiles during acute inflammation. As an example of acute wound fluid, we used mastectomy fluid. Gelatinase patterns observed in samples from ten patients were all similar, and Figure 2 shows representative zymograms for two subjects whose wound fluid samples were obtained days 1 and 2 after surgery. The group of four gelatinase bands that were observed in blood-derived serum were also the predominant gelatinases in mastectomy wound fluid: that is, MMP-2, MMP-9, and the 130-kDa and 225-kDa bands believed to be MMP-9–containing complexes.

Enzymatic activity on zymograms was quantified by scanning densitometry of the bands and measuring the area under the peaks. One zymogram unit was defined as activity corresponding to an area of 1 mm^2. Figure 3 shows a plot of MMP-2 and MMP-9 gelatin-

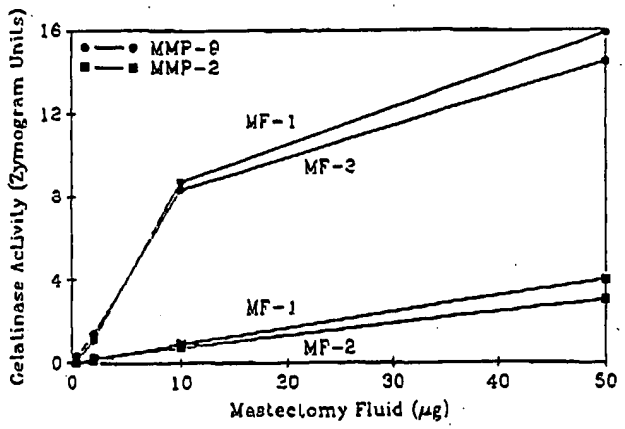

Figure 3. Analysis of zymograms by scanning densitometry. Samples of mastectomy wound fluid as indicated were subjected to zymography. Zymograms were photographed to obtain negatives, and the negatives were analyzed using a scanning laser densitometer (LKB Ultrascan). Areas corresponding to the MMP-2 and MMP-9 bands on the negatives—clear zones on the originals—were quantified with a digitizing pad. One zymography using equals an area of 1 mm^2. Data shown are for two different mastectomy fluid samples.

Table I. Concentration of Gelatinases in Serum and Wound Fluid[a]

Experiment	Sample	Protein (μg)	Gelatinase Activity (Zymography Units) 72 kDa (MMP-2)	94 kDa (MMP-9)	130 kDa	225 kDa
1	PDS	75	4.5 ± 0.4[b]	0.6 ± 0.3		
	BDS	75	4.4 ± 0.3	5.0 ± 2.0		
2	MF	75	8.6 ± 2.9	23.3 ± 3.8		
3	PDS	75	1.6	0.3	0.1	0.2
	BDS	75	1.4	2.1	0.2	0.8
	MF	75	5.2	19.6	8.2	4.5
4	MF-1	2	0.2	1.2	0.2	0.2
	CWF-DA-1	2	0.6	5.1	0.4	0.3
	CWF-DA-2	2	1.0	18.5	5.4	0.8

[a] Gelatinase activity was determined as described in the legend to Fig 3. Experiment 1: samples (75 μg) of PDS and BDS from four different subjects. Experiment 2: samples (75 μg) of five different mastectomy wound fluids. Experiment 3: samples (75 μg) of PDS, BDS, and mastectomy wound fluid. Experiment 4: samples (2 μg) of mastectomy wound fluid and chronic wound fluid.

[b] Mean ± SD.

ase activity versus concentration of mastectomy fluid for two different mastectomy fluid samples. The results show a relatively linear dose-response relationship for activity measured by densitometry, particularly at enzyme activities less than 10 units.

Table I, experiment 1 shows another experiment in which 75-μg samples of PDS and BDS from four different subjects were analyzed. As was evident from Fig 1, MMP-2 levels were similar to PDS and BDS. MMP-9 levels were about ninefold higher in BDS compared with PDS. Table I, experiment 2 shows a similar analysis for five different mastectomy samples. The results in Fig 3 and Table I, experiments 1 and 2, indicate that, for samples analyzed on the same zymogram, densitometric analysis provides a high degree of precision. Nevertheless, there was some variability in the results from day to day, partly as a result of slight changes in the gels, but especially because the gels were photographed to optimize visualization of the bands.

Comparison of experiments 1 and 2 in Table I show that MMP-2 levels were about twofold higher in mastectomy fluid than in PDS and BDS, and MMP-9 levels were at least fivefold higher than in PDS. Considering the results in Fig 3, the latter value is a conservative estimate. When representative samples of PDS, BDS, and mastectomy fluid were analyzed on the same zymogram (Table I, experiment 3), the levels of MMP-2 were about threefold higher in mastectomy fluid than in BDS or PDS, whereas the levels of MMP-2 or MMP-9–containing complexes were more than tenfold higher in mastectomy fluid. These data provide direct evidence for increased MMP-9 levels during the acute inflammatory response *in vivo*, which most likely resulted from release of MMP-9 by neutrophils and monocytes that migrated into the wound bed [23,24]. On the other hand, MMP-2 remained closer to basal levels, as would be expected at the early stages of repair before granulation tissue formation, assuming that fibroblasts are the major source of this enzyme [25].

Gelatinase Activity in Chronic Wound Fluid When the gelatinase profiles of wound fluid obtained from six patients with chronic leg ulcers were compared with serum or mastectomy fluid, there were several significant differences. First, there was not a single proteinase pattern common to all of the patients. Figure 4 shows samples collected from four patients, two in Dallas (DA) and two in New York City (NY). Four of the six patients had gelatinase profiles similar to DA-1 and NY-1, but DA-2 and NY-2 were unique. Consistent with previous studies [4], changes in gelatinase levels occurred locally, not systemically, because blood samples collected from the Dallas patients showed only baseline gelatinase levels (data not shown).

Second, the gelatinase profiles of chronic wound fluid were much more complex than those of mastectomy wound fluid or serum. Rather than four gelatinase bands, there were seven or more. Of particular interest were the bands that appeared just beneath MMP-2 and MMP-9, ~8 kDa smaller (see also Fig 5). This pattern would be expected if the proenzyme forms of the enzymes had been cleaved to the slightly smaller activated forms. Chronic wound fluids DA-2 and NY-2 also contained several gelatinases less than 45 kDa that we have yet to identify.

Finally, the gelatinase levels in chronic wound fluid were higher than in mastectomy wound fluid. This difference is illustrated by Fig 5, which shows a zymogram of serial dilutions of two chronic wound fluid samples compared to each other and to mastectomy wound fluid. Table I, experiment 4 shows a comparison of gelatinase activity levels in samples diluted to 2 μg. MMP-2 levels in chronic wound fluid were three- to fivefold higher than in mastectomy fluid, and MMP-9 levels were five- to twentyfold higher than in mastectomy fluid. In other experiments, analysis of serial dilutions of chronic wound fluids NY-1 and NY-2 indicated that MMP-2 and MMP-9 levels in these samples were intermediate between DA-1 and DA-2 (data not shown).

Table II. Effect of Proteinase Inhibitors on Gelatin Zymographic Activity[a]

Inhibitor	Specificity	Concentration	Effect on Zymogram
Ethylenediaminetetraacetic acid	Metallo	10 mM	Complete inhibition
1,10-phenanthroline	Metallo	5 mM	Complete inhibition
AEBSF	Serine	10 mM	No effect
Phenylmethylsulfonylfluoride	Serine	0.2 mM	No effect
Tosyl lysyl chloromethyl ketone	Serine	100 μM	No effect
Tosyl phenylalanyl chloromethyl ketone	Serine	100 μM	No effect
Aprotinin	Serine	20 μM	No effect
Leupeptin	Serine/cysteine	20 μM	No effect
Pepstatin A	Aspartic	20 μM	No effect

[a] Samples of serum or mastectomy fluid (75 μg) or chronic wound fluid (2–10 μg) were subjected to gelatin zymography. Inhibitors were added to the overnight reaction buffer at the concentrations indicated. In some experiments, the inhibitors AEBSP and aprotinin were added to the wash buffer as well as the reaction buffer. AEBSF was purchased from Calbiochem, and all other inhibitors were obtained from Sigma Chemical Co.

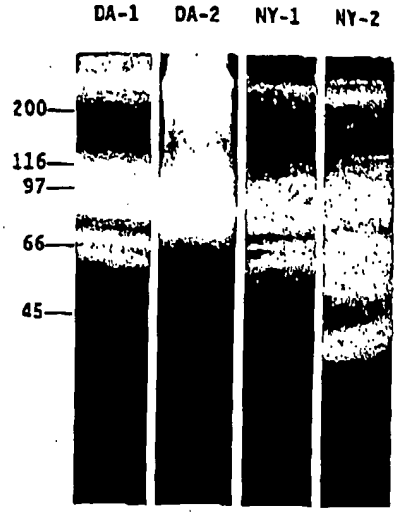

Figure 4. Gelatinase activity in chronic wound fluid. Samples (75 μg) of chronic wound fluid were subjected to gelatin zymography. Results shown are from four different subjects, two whose wound fluid was collected in Dallas (DA) and two whose wound fluid was collected in New York (NY). Gelatinase profiles observed with DA-1 and NY-1 were typical of six of the eight subjects studied. Chronic wound fluid contained several gelatinases not found in mastectomy fluid including components ~8 kDa smaller than MMP-2 and MMP-9, possibly corresponding to activated MMP-2 and MMP-9 enzymes.

Effect of Proteinase Inhibitors on Wound Fluid Gelatinases

The above results showed that chronic wound fluid contained higher levels of gelatinases than mastectomy wound fluid and also contained what appeared to be activated forms of gelatinase that were absent from mastectomy wound fluid. To confirm that these gelatinases were metalloproteinases, enzyme inhibitor studies were performed. Table II summarizes the reagents that were tested in these studies. Addition of the metalloproteinase inhibitors ethylenediaminetetraacetic acid or 1,10-phenanthroline to the zymography reaction buffer resulted in a complete inhibition of all zymography bands detected in serum, mastectomy wound fluid, and chronic wound fluid. On the other hand, addition of other inhibitors had no detectable effect, even if they were added to both the wash solution and reaction buffer. In control experiments, aminoethylbenzene sulfonylfluoride (AEBSF) and aprotinin were shown to inhibit zymography bands observed with trypsin and plasmin, whereas trypsin activity was unaffected by 1,10-phenanthroline (data not shown).

DISCUSSION

We used gelatin zymography [17] to compare metalloproteinase profiles in chronic wound fluid with acute wound fluid and serum. Analysis of wound fluid as an indicator of the wound environment is a useful method for studying chronic ulcers because the fluid can be obtained non-invasively by briefly covering the patient's ulcer with an occlusive dressing. Although there may be some molecules selectively retained in the wound bed, the overall polypeptide profiles of chronic and acute wound fluid and serum were found to be similar [3,4]. Moreover, even large matrix molecules such as cellular fibronectin diffused through the wound bed into the wound fluid [4].

Serum was used to assess baseline levels of proteinases that might occur in wound fluid, and mastectomy fluid was used to assess changes in proteinases associated with the acute inflammatory response. Plasma-derived serum contained primarily MMP-2 (72-kDa gelatinase) consistent with a previous report [21]. Blood-derived serum contained MMP-2 and in addition MMP-9 and MMP-9–containing complexes, indicating that cellular release of this enzyme occurred during clotting, probably from neutrophils and monocytes [23,24]. Acute wound fluid (mastectomy fluid) contained the same group of four gelatinase bands as blood-derived serum but at higher levels, approximately threefold higher MMP-2 and more than tenfold higher MMP-9. Nevertheless, only proenzyme forms of MMP-2 or MMP-9 were detected. Therefore, although the initial inflammatory response after wound healing resulted in increased proenzyme accumulation, the proenzymes did not appear to become activated. It should be noted that our identification of MMP-2 and MMP-9 has been based solely on zymographic results, and studies with specific antibody reagents have yet to be carried out.

Higher levels of MMP-9 compared to MMP-2 after initial wounding are consistent with inflammatory cells as the major source of MMP-9 and fibroblasts as the major source of MMP-2 [23–25]. Assuming that MMP-9 in the wound bed comes mostly from inflammatory cells, the MMP-9/MMP-2 ratio may provide an index of inflammation. In rabbit corneal keratectomy wounds, which involve a minimal inflammatory response, the MMP-9/MMP-2 ratio remained quite low [26]. Our studies showed that the MMP-9/MMP-2 ratio was lowest in PDS, intermediate in mastectomy fluid, and highest in chronic wound fluid. Therefore, chronic wounds may represent a persistent inflammatory state, which could have a variety of causes, including the presence of focal bacterial colonies in the wound bed [27–29].

Metalloproteinase levels in chronic wound fluid were much higher than in mastectomy fluid or serum. Moreover, unlike mastectomy fluid, the gelatinase profiles of chronic wound fluid showed several bands in addition to the proenzyme forms of the metalloproteinases. Although we have studied only a small patient population so far, these differences observed were found with patients from different geographic locations and with wound fluid samples collected by different investigators.

Gelatinase bands that appeared ~8 kDa smaller than MMP-2 and MMP-9 would be expected if the proenzyme forms of the enzymes had been cleaved to the slightly smaller activated enzyme species. The *in vivo* mechanism of activation of these collagenases is as yet unknown [14,15]. Plasmin, which has been suggested to initiate activation of interstitial collagenase and stromolysin [30], probably is not responsible for activation of the MMP-2 and MMP-9 [31]. A variety of serum and tissue proteinase inhibitors normally regulate proteinase activity [12–15]. Therefore, metalloproteinase activation in chronic wounds could result from increased levels of an activator or decreased levels of an inhibitor. Future studies on chronic wound fluid should provide an opportunity to learn more about the *in vivo* regulatory mechanisms for MMP-2 and MMP-9.

The increased level and activation of metalloproteinases in

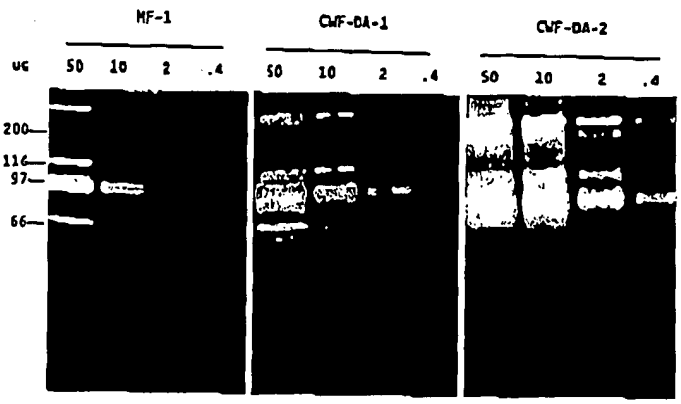

Figure 5. Comparison of gelatinase activities in mastectomy and chronic wound fluids. Serially-diluted samples of mastectomy wound fluid and chronic wound fluids DA-1 and DA-2 (50–0.4 μg) were subjected to gelatin zymography. Comparison of the profiles at different sample sizes showed that gelatinase levels were higher in chronic wound fluids than in mastectomy wound fluid. Also, gelatinase levels in chronic wound fluid DA-2 were higher than in DA-1.

chronic wound fluid may be responsible, at least in part, for degradation of adhesion proteins that occurs in chronic wound fluid but not in acute wound fluid [3,4]. Consistent with this idea, the proteinase levels in chronic wound fluid DA-2 were higher than DA-1, and fibronectin and vitronectin were more completely degraded in DA-2 wound fluid than in DA-1 wound fluid [4]. Recently, we also have observed a correlation between high gelatinase levels and the extent of adhesion protein degradation in acute burn fluid (Grinnell and Zhu, unpublished observations).

Because fibronectin and other adhesion proteins are important for normal wound repair [5], degradation of the adhesion proteins by proteinases in chronic ulcers could contribute to poor healing of these wounds. Moreover, fibronectin fragments may help maintain high proteinase levels by stimulating neutrophil degranulation [32] and fibroblast secretion of metalloproteinases [33]. Also, some fibronectin fragments exhibit endogenous proteinase activity towards gelatin and laminin [34]. Finally, elevation of proteinases in wound fluid could interfere with normal healing not only by degrading adhesion proteins, but also by degrading other factors necessary for repair. Therefore, chronic tissue degradation caused by an environment containing high levels of activated metalloproteinases may play a major role in failed wound closure.

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